Double identity for proteins of the Bcl-2 family

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Bcl-2 is an oncogenic protein that acts by inhibiting programmed cell death. The mechanisms used by this and related anti-apoptotic proteins to protect cells from cytotoxic stimuli are now emerging, with the discovery that Bcl-2 can function both as an ion channel and as an adaptor or docking protein.

Since its discovery over ten years ago as an oncogenic protein involved in human tumours[1], Bcl-2 has defied attempts to determine the biochemical basis for its potent anti-apoptotic action. Recent findings, however, are beginning to reveal details of the mechanisms by which Bcl-2 and its homologous proteins, such as Bcl-X_L, suppress cell death. A picture is emerging of a complicated protein, which in some ways resembles p53 in that it has multiple independent functions, rather like a Swiss army knife.

Although Bcl-2 family proteins lack significant amino-acid sequence homology with other proteins, the three-dimensional structure of Bcl-X_L[2] reveals a striking similarity to the pore-forming domains of certain bacterial toxins that act as channels for either ions or proteins. The closest in structure to Bcl-X_L are diphtheria toxin, which transports a fragment of the toxin across the cell membrane, and the bacterial pore-forming colicins, which kill *Escherichia coli* by forming non-selective ion channels. If function follows form, the obvious prediction is that Bcl-2 family proteins can function as channels for ions, proteins, or both.

Circumstantial evidence from cell transfection studies indicated that Bcl-2 might have a membrane transport function, with reported effects on Ca^{2+} flux and protein translocation across some of the intracellular membranes where Bcl-2 and its homologues are localized, namely the outer mitochondrial membrane, the endoplasmic reticulum (ER) and the nuclear envelope[3–5]. Now, direct evidence of ion-channel activity has been obtained from experiments in which the effects of recombinant Bcl-2 or Bcl-X_L in synthetic lipid membranes were studied by using single-channel recordings from planar bilayers and by other approaches[6,7]. The mechanism by which Bcl-2 and Bcl-X_L create channels in membranes has not been explored in detail, but preliminary indications are that at least some aspects of the process may be similar to the bacterial toxins. For example, the structure of Bcl-X_L reveals a compact seven α-helical bundle, at the centre of which lies a hairpin comprised of two long hydrophobic core α-helices surrounded by five amphipathic α-helices[2]. By analogy to the bacterial toxins, the two core α-helices (α_5 and α_6) presumably insert perpendicularly across the lipid bilayer, with the protein undergoing profound conformational changes, perhaps opening like an umbrella, with the surrounding amphipathic helices folding upwards and resting on top of the membrane. Consistent with this idea, removal of α_5 and α_6 from Bcl-2 prevents channel formation *in vitro* and abolishes its anti-apoptotic effect in cells[7].

Details about the structures of Bcl-2 and Bcl-X_L channels are still to be resolved and already controversy has developed about the likely size (diameters) of these channels. For example, it has been reported that Bcl-2 and Bcl-X_L form discrete ion channels *in vitro* which mostly assume a closed conformation, open only sporadically, and yield conductances ranging from ~20 to ~300 picosiemens (pS)[6,7]. However, evidence has been found for larger pores under some conditions, which assume a mostly open conformation and produce channels with conductances >1 nS (S. Korsmeyer, personal communication). Related to the issue of pore diameter is the question of the stoichiometry of these channels. Although the predicted membrane-spanning α_5 and α_6 of Bcl-2 and Bcl-X_L are mostly hydrophobic, as required for insertion into membranes, one face of the helices has hydrophilic residues, which presumably line the aqueous lumen of the channels. By analogy to other natural and synthetic α-helix-type channels (ref. 8), it follows that the membrane-inserted helices from two or more Bcl-2/Bcl-X_L molecules should associate to form a ring that creates an aqueous lumen. How many molecules of Bcl-2 or Bcl-X_L assemble in membranes to create channels is not known, but the existence of multiple Bcl-2/Bcl-X_L conductance states suggests a range of possibilities, at least *in vitro*[6,7]. There is evidence that Bcl-X_L can function as a monomer, but so far we do not have the methodology to determine the oligomerization state of this protein when it is integrated into membranes and so cannot discount the channel hypothesis. The challenge now is to demonstrate that Bcl-2/Bcl-X_L channels exist *in vivo* and to determine their structures.

Complicating the question of the biological significance of these findings are data indicating that the pro-apoptotic Bcl-2 family member Bax, as well as Bcl-2 and Bcl-X_L, have channel activity *in vitro* (J. C. Martinou, personal communication; S. Schendel, Z. Xie and J.C.R., unpublished results). As Bax can heterodimerize with either Bcl-2 or Bcl-X_L when they are in their compact α-helical-bundle conformations in aqueous environments, these proteins may produce heteromeric channels when integrated into membranes. Alternatively, their heterodimerization may prevent Bcl-2 and Bax from undergoing the conformational changes required for integration into membranes, thus preventing channel formation. Both Bcl-2 and Bax might therefore form cytotoxic channels in cells, with Bcl-2/Bax heterodimerizaiton nullifying channel activity and thus promoting cell survival. This idea predicts that the Bcl-2 : Bax ratio is critical, consistent with the finding that excess Bcl-2 paradoxically promotes cell death in some circumstances[9] and with evidence that Bax and Bak unexpectedly exhibit cytoprotective functions in some cellular contexts[10,11]. Or Bcl-2/Bcl-X_L and Bax might have different selectivities, providing conduits for different ions or proteins that have opposing effects on apoptosis—this possibility could be tested by mutagenesis in which the presumed channel-forming fifth and sixth helices of these proteins are swapped.

Although at present we have no direct evidence for *in vivo* channel formation, the fact that Bcl-2 family proteins can form channels *in vitro* suggests that they may participate in some of the cellular phenomena recently associated with apoptosis, particularly mitochondrial permeability transition ('megapore' opening) and release of apoptogenic protease activators (cytochrome *c* and apoptosis-inducing factor (AIF)) from mitochondria. Mitochondrial permeability transition involves the opening of a large (~1.3 nS, ~2.9 nm) channel in the inner membrane of the mitochondrion[12,13]. This occurs almost universally during apoptosis and has repercussions that may contribute to the induction of cell death, including

the generation of oxygen free radicals, dumping of stored Ca^{2+} into the cytosol, and the release of mitochondrial proteins into the cytosol in order to activate the cysteine proteases (caspases) that are the terminal effectors of apoptosis[14]. Overexpression of Bcl-2 inhibits mitochondrial permeability transition, whereas Bax overexpression induces it[15,16]. Bcl-2 is found on the outer mitochondrial membrane and is enriched at the contact sites where the inner and outer membranes abut. As some proteins that regulate the megapore reside in the outer membrane, Bcl-2 and Bax could somehow modulate or participate in megapore formation, possibly by controlling ion fluxes that influence the opening and closing of the megapore. On the other hand, some studies suggest that caspase-activating proteins, in particular cytochrome *c*, can leave the mitochondrial intermembrane space through the outer membrane and enter the cytosol where the cell-death proteases are found, irrespective of mitochondrial permeability transition[17,18]. If so, channels must exist in the outer mitochondrial membrane that control the release of cytochrome *c* during apoptosis. It remains to be determined whether Bax can create such channels and whether the suppression of cytochrome *c* release by Bcl-2 reflects an ability of Bcl-2 either to plug Bax channels or to transport cytochrome *c* back into mitochondria[17,18].

Although the new-found channel activity of Bcl-2 family proteins may prove to be crucial to their function as regulators of cell life and death, these proteins have other important ways of controlling apoptosis. In this regard, Bcl-2 or Bcl-X_L has been reported to bind (or at least coimmunoprecipitate with) eleven proteins, including the protein kinase Raf-1, the protein phosphatase calcineurin, the GTPases R-Ras and H-Ras, the p53-binding protein p53–BP2, the prion protein Pr-1, and several proteins with unknown functions, including CED-4, BAG-1, Nip-1, Nip-2 and Nip-3 (reviewed in ref. 19, and see below). Conversely, the pro-apoptotic protein Bax apparently does not interact with these proteins, suggesting an important difference between the death-suppressing and death-promoting members of the Bcl-2 family.

Although the functional significance of these interactions of proteins with Bcl-2 is unclear, recent discoveries are reducing our ignorance while also raising many questions. The developing theme is that Bcl-2 and its anti-apoptotic homologues, which are normally anchored in intracellular membranes (by virtue of a stretch of hydrophobic residues at their carboxy termini) but oriented towards the cytosol, act at least in part as adaptor or docking proteins which pull other proteins out of the cytosol, either sequestering them to the membrane alongside Bcl-2 (and probably inactivating them) or targeting them for interaction with other membrane-associated proteins.

Of the Bcl-2/Bcl-X_L-binding proteins, CED-4 or its functional equivalents in mammalian cells is probably the most important. CED-4 is a pro-apoptotic protein from the nematode *Caenorhabitis elegans*[20]. In a genetic sense, CED-4 functions upstream of the worm homologue of the caspases (CED-3) but downstream of the worm version of Bcl-2 (CED-2) (ref. 21). Although they are functionally connected, it was unclear whether CED-9, CED-4 and CED-3 were physically linked or whether other proteins might be required to create a bridge between them. This speculation was stopped by the recent demonstration that CED-9 as well as Bcl-X_L can bind to worm CED-4, which in turn binds to CED-3 or other caspases[22–24]. Indeed, CED-9, CED-4 and CED-3 can form a trimolecular complex[23]. The mammalian homologue of CED-4 remains at large, but experiments demonstrating co-immunoprecipitation of Bcl-X_L with caspases such as FLICE (caspase-8) and ICE (caspase-1) in human cells indicate that CED-4 homologues may exist in higher organisms. A race is now underway to clone mammalian CED-4 and to define its three-dimensional structure, as well as the mechanism by which it may activate the caspases. In the worm, two forms of CED-4 have been described that arise through alternative splicing of messenger RNA, with the shorter CED-4 promoting apoptosis and the longer CED-4 promoting cell survival[25]. If this is a sign of things to come in mammalian species, then we can expect an additional level of complexity at the CED-4-dependent step of the cell-death pathway.

Another burning question is how do anti-apoptotic Bcl-2 family proteins such as CED-9 and Bcl-X_L interfere with CED-4 function? Overexpression of worm CED-4 in mammalian cells induces apoptosis in a CED-9- and Bcl-X_L-suppressible manner. CED-4 is normally found in the cytosol, but when co-expressed with CED-9 or Bcl-X_L, it is sequestered to the intracellular membranes where CED-9 and Bcl-X_L are located[24]. Conversely, when pro-apoptotic members of the Bcl-2 family such as Bax, Bak and Bik are co-expressed with Bcl-X_L, they dimerize with Bcl-X_L, thereby displacing CED-4 (Fig. 1). Thus, it appears that anti-apoptotic Bcl-2 family proteins pull CED-4 out of the cytosol, but the question of exactly how they inhibit CED-4 remains unanswered.

Interactions with other proteins may not be as fundamental to Bcl-2's function as CED-4 binding, but they may account for other activities previously associated with Bcl-2 and provide insight into how Bcl-2 family proteins are regulated. For example, Bcl-2 (but not Bax) can associate with Raf-1, pulling this kinase from the cytosol into proximity with the Bcl-2 on the mitochondrial membrane. Once there, Raf-1 induces phosphorylation of BAD, a pro-apoptotic Bcl-2 family protein that abrogates the cytoprotective functions of Bcl-2 and Bcl-X_L by heterodimerizing with them. The phosphorylated BAD (which unlike Bcl-2 has no membrane-anchoring domain) then dissociates from Bcl-2/Bcl-X_L, moving into the cytosol in a complex with 14-3-3, where it cannot interfere with Bcl-2/Bcl-X_L. Although BAD is one potential substrate of Bcl-2/Raf-1,

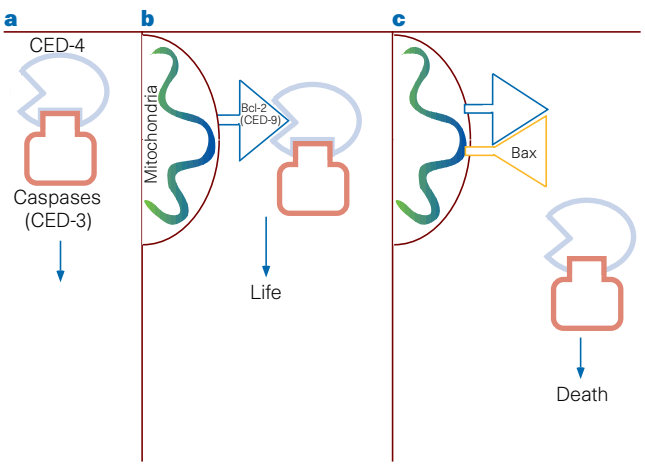

Figure 1 Model for CED-4-mediated bridging of Bcl-2/Bcl-X_L to caspases. **a**, CED-4 binding to cell-death proteases (caspases) such as CED-3 promotes apoptosis. **b**, Bcl-2, tethered to mitochondrial and other intracellular membranes, binds to CED-4, pulling it from the cytosol and presumably preventing activation of caspases. **c**, By heterodimerizing with Bcl-2, pro-apoptotic proteins such as Bax displace CED-4 and presumably allow it to return to the cytosol and to activate proteases.

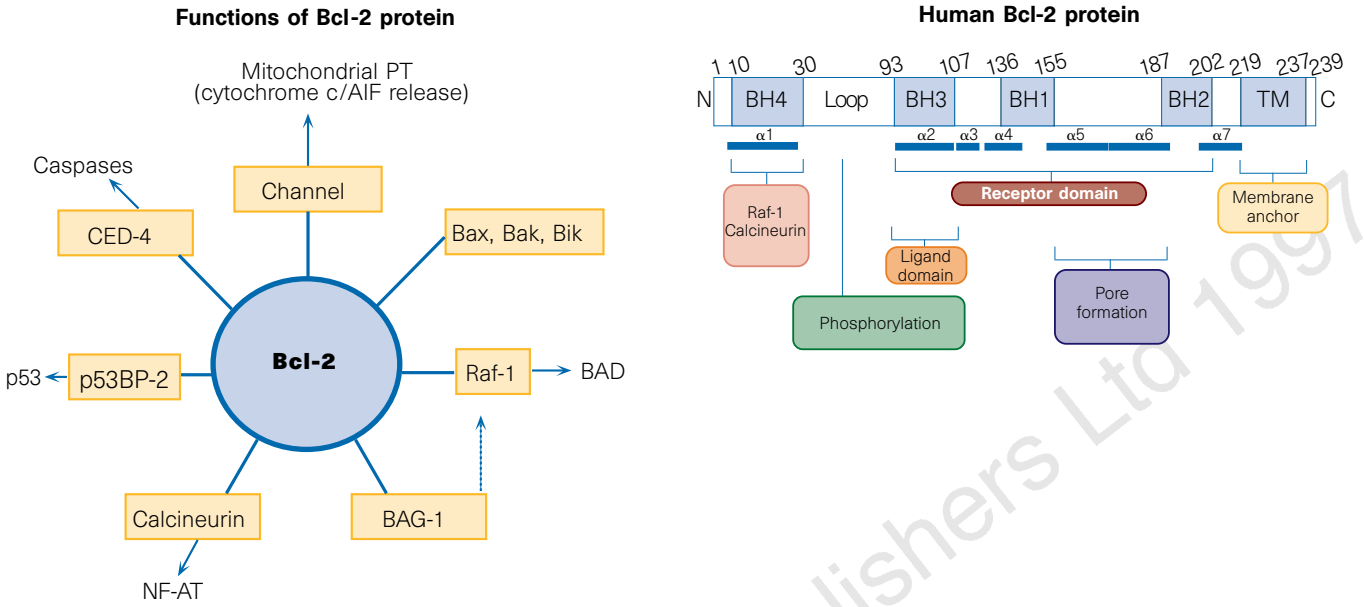

Figure 2 Multifunctional Bcl-2 protein. Left, in addition to channel activity which may directly or indirectly influence mitochondrial megapore opening or release of cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria, Bcl-2 or Bcl-X_L also binds to several proteins that can participate in cell-death regulation. CED-4 provides a physical connection to the cell-death proteases (caspases). See text for details. Right, the topology of the human Bcl-2 protein is depicted, showing the locations of the Bcl-2 homology (BH) domains, the predicted α-helical segments, and the transmembrane (TM) anchor. Binding of Raf-1 and calcineurin is BH4-dependent (first α-helix). A long flexible loop between the first and second α-helices is required for Bcl-2 phosphorylation and may represent a negative-regulatory domain. The BH3 domain (second α-helix) plays the role of 'ligand' during dimerization of Bcl-2 family proteins, whereas the combination of the BH1, BH2 and BH3 domains appears to be required for forming the hydrophobic groove into which the BH3 domain inserts. The fifth and sixth α-helices are predicted to participate in channel formation, presumably by penetrating lipid bilayers.

presumably others exist as artificially targeting active Raf-1 to mitochondria can also suppress apoptosis in cells that do not contain BAD protein[26,27]. Another Bcl-2-binding protein, BAG-1, can also interact with and activate Raf-1. Thus, BAG-1 probably collaborates with Bcl-2, providing a mechanism for activating Raf-1 locally at mitochondrial and other Bcl-2-containing membranes in a Ras-independent manner[28].

Little is known about the regions of the Bcl-2 protein needed for interaction with other proteins. However, the three-dimensional structure of Bcl-X_L in its compact, non-membrane-integrated conformation reveals the presence of a hydrophobic groove on the surface of the protein, which is partly formed by the well conserved BH1, BH2 and BH3 domains[2]. The second amphipathic helix of dimerized Bcl-2 family proteins inserts into this groove, like a peptide ligand binding to a receptor[29,30]. As the BH3 domain corresponds to this inserting α-helix (ligand) but also forms part of the surface pocket (receptor), the implication is that Bcl-2 family proteins assume at least two conformations even in aqueous environments, with one dimerizing partner playing the role of the receptor and the other the ligand. Where CED-4 binds on Bcl-2/Bcl-X_L is unknown, but this surface pocket is a likely candidate, given that some pro-apoptotic Bcl-2 family proteins such as Bik can displace CED-4 from Bcl-X_L and yet have only the BH3 domain (thus limiting their role to that of the ligand). Such 'BH3 only' proteins evidently lack the hydrophobic helices required for membrane integration, suggesting that they can induce apoptosis without forming channels in membranes.

The so-called 'BH4' domain near the N terminus of Bcl-2 is probably not required for binding to CED-4 but is needed for interaction with Raf-1 (refs 25, 26). This domain represents the first α-helix in Bcl-X_L (ref. 2) and is present in all anti-apoptotic Bcl-2 family proteins but absent from nearly all of the pro-apoptotic proteins, implying that Bcl-2/Bcl-X_L evolved an additional domain that provides a means for communicating with Raf-1. The Ca^{2+}-dependent protein phosphatase calcineurin also binds to the BH4 domain of Bcl-2 (ref. 31). Overexpression of active calcineurin induces apoptosis in a Bcl-2-suppressible manner[32], suggesting a role for this phosphatase during Ca^{2+}-mediated apoptosis. Bcl-2 causes a redistribution of calcineurin from the cytosol to intracellular membranes, preventing interaction with phosphorylated NF–AT or other substrates of calcineurin in the cytosol. How calcineurin induces apoptosis is unclear, but dephosphorylation of BAD is one possibility. The Bcl-2/calcineurin interaction may also be relevant to the inhibition by Bcl-2 of cell proliferation and of translocation of NF-AT into the nucleus[33,34], given that NF-AT-inducible genes are important for proliferation in some types of cell such as lymphocytes. Although it is not fundamental to its function as a suppressor of apoptosis[35], the slowing of entry of quiescent cells into the cell cycle by Bcl-2 could provide additional cytoprotection, because proliferating cells can be more vulnerable to apoptotic stimuli. With kinases and phosphatases hovering around Bcl-2, it is notable that Bcl-2 can be phosphorylated and that this post-translational modification may inactivate Bcl-2 (refs 36, 37) (Fig. 2).

In addition to CED-4, BAG-1, Raf-1 and calcineurin, Bcl-2 can bind to a p53-binding protein, p53-BP2, conceivably explaining how overexpression of Bcl-2 can interfere with translocation of p53 from the cytosol into the nucleus[5,38]. Immunoelectron microscopy indicates that Bcl-2 is associated with nuclear pore complexes[39], an ideal situation for catching proteins as they cross the nuclear envelope.

The available data thus suggest that Bcl-2 has two separate functions as a channel protein and as an adaptor/docking protein (Fig. 2). Although arguments could be raised against both models on the basis that truncation mutants of Bcl-2 without their carboxy-terminal membrane-anchoring domain retain anti-apoptotic function, these mutant proteins still associate with intracellular membranes, probably by virtue of interaction with endogenous Bcl-2 family proteins. The channel and adaptor protein activities may be mutually exclusive in some circumstances, because for channel formation Bcl-2 must integrate into membranes and drastically

alter its conformation, which might prevent it from interacting with CED-4 or other key proteins. Conversely, some protein interactions may be assisted by this integration, for example those of the BH4 domain with Raf-1 and calcineurin. Which of the two functions will prove to be more important? The genetic data from *C. elegans* indicate that CED-4 is likely to be critical for Bcl-2 function, enabling Bcl-2 to regulate the caspases. Moreover, the finding that CED-9 is not required for survival of worms that lack CED-3 or CED-4 argues that channel formation is unnecessary[21]. However, Bax can induce cell death by means that do not depend on caspases, apparently by triggering mitochondrial permeability transition[16].

The dual function of Bcl-2 could explain how Bcl-2 can inhibit both apoptosis and (in some cases) necrosis[40] by interacting with CED-4 to modulate caspases and regulating motochondrial permeability transition through its ion-channel activity, respectively. Interaction of Bcl-2 with accessory proteins such as Raf-1, calcineurin, BAG-1 and p53-BP2 will likewise find their niches as additional cell-type- or circumstance-specific embellishments on the theme of Bcl-2/Bcl-X_L as adaptor/docking protein, probably serving as supporting actors in a play in which channel activity and CED-4 binding star as the principal protagonists. □

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